

Adenovirus-Delivered Antisense RNA and shRNA Exhibit Different Silencing Efficiencies for the Endogenous Transforming Growth Factor- β (TGF- β) Type II Receptor

MIROSLAVA OGORELKOVA,¹ JOHN ZWAAGSTRA,¹ SEYYED MEHDY ELAHI,¹
CHRISTEL DIAS,¹ CLAIRE GUILBAUT,¹ RITA LO,¹ CATHY COLLINS,¹ MARIA JARAMILLO,¹
ALAKA MULLICK,¹ MAUREEN O'CONNOR-McCOURT,¹ and BERNARD MASSIE^{1,2,3}

ABSTRACT

Gene silencing is an essential tool in gene discovery and gene therapy. Traditionally, viral delivery of antisense RNA and, more recently, small interfering RNA (siRNA) molecules in the form of small hairpin RNAs (shRNA) has been used as a strategy to achieve gene silencing. Nevertheless, the enduring challenge is to identify molecules that specifically and optimally silence a given target gene. In this study, we tested a set of adenovirus-delivered antisense RNA fragments and adenovirus-delivered shRNA molecules for their ability to target human transforming growth factor- β type II receptor (TGF β RII). We used a dicistronic reporter, consisting of the coding sequences for TGF β RII and green fluorescent protein (GFP) to screen for optimal silencing agents targeting TGF β RII. Our results show, for both antisense RNA and shRNA molecules, that their effectiveness in the GFP screen correlated directly with their ability to reduce exogenously expressed TGF β RII. Unexpectedly, the antisense RNAs were unable to silence endogenous TGF β RII. In contrast, the shRNAs were able to silence endogenous TGF β RII. The shRNA that demonstrated the most pronounced effect on the dicistronic TGF β RII/GFP reporter reduced endogenous TGF β RII protein expression by 70% in A549 cells and reduced TGF β signaling by >80% in HeLa cells.

INTRODUCTION

SPECIFIC DOWNREGULATION of gene expression is an invaluable tool for studying gene function, gene discovery, and targeting disease-related genes using gene therapy. Vector-based expression of long-chain antisense RNAs has been one of the most commonly applied gene-silencing techniques over the past decade (reviewed in Weiss et al., 1999). Numerous studies on the determinants of antisense functionality have been carried out (reviewed in Sczakiel, 1997), and computational algorithms predicting RNA structure and annealing have been developed for rational design of antisense target sequences

(Patzel and Sczakiel, 1998; Lehmann et al., 2000; Ding and Lawrence, 2001). Nevertheless, the fact remains that not all antisense RNAs designed against a particular target have an antisense effect, and selection of efficient antisense RNAs is largely a matter of trial and error.

More recently developed techniques for specific gene downregulation are based on the phenomenon of RNA interference (RNAi) (reviewed in Paddison and Hannon, 2002; Shi, 2003). Transfection of short 21–23-nucleotide (NT) double-stranded RNA (dsRNA) duplexes, known as small interfering RNAs (siRNAs), can induce specific and potent gene silencing in mammalian cells (Elbashir et al., 2001). Moreover, siRNAs can be transcribed using

¹Biotechnology Research Institute, Montréal, Québec, Canada H4P 2R2.

²INRS-IAF, Université du Québec, Laval, Québec, Canada H7N 4Z3.

³Département de microbiologie et immunologie de l'Université de Montréal, Montréal, Québec, Canada H3C 3J7.

Pol III promoters as stem-loop structures (short hairpin RNAs [shRNAs]) that can effectively initiate RNAi (reviewed in Paddison and Hannon, 2002; Shi, 2003), and shRNA expression cassettes have been adapted to retroviral, lentiviral, and adenoviral vectors (Abbas-Terki et al., 2002; Brummelkamp et al., 2002; Shen et al., 2003). As with antisense RNA technology, not all siRNAs are equally efficient in initiating gene silencing (Elbashir et al., 2001; Holen et al., 2002; Lee et al., 2002; Miyagishi et al., 2003; Yu et al., 2002). Currently, there are no reliable ways to predict siRNA efficacy, and testing several siRNAs against a given target is necessary to identify the ones with the strongest inhibitory effect (Shi, 2003).

A major consideration in optimizing the utility of antisense RNA and siRNA is to accurately assess their silencing effects on an endogenous target gene, in particular when the gene is expressed in poorly transfectable cell lines or when the gene product is not known or is difficult to detect. The use of reporter transgene targets may, therefore, facilitate the selection of efficient silencing molecules. There are only limited reports about the feasibility of this approach, and little is known about its reliability. The efficacy of antisense oligodeoxynucleotides (t'Hoen et al., 2002) and antisense RNA (Ohkawa and Taira, 2000) has been tested on reporter constructs consisting of the coding sequence of the gene of interest fused to the coding sequence of green fluorescent protein (GFP). However, the efficacy on the corresponding endogenous targets was not assessed in these studies. Similarly, transfected siRNAs have been reported to downregulate the expression of both target/reporter gene fusion transgenes and endogenous genes (Holen et al., 2002; Kumar et al., 2003; Nagy et al., 2003). To date, however, viral delivery and screening with such a reporter has not been addressed.

The transforming growth factor- β (TGF- β) family of cytokines regulates a wide variety of biologic processes, such as proliferation, differentiation, and cell death (Lawrence, 1996; Massagué et al., 2000). TGF- β plays a fundamental role in multiple diseases, including cancer and fibrosis (Blobe et al., 2000; Massagué et al., 2000). In the present study, we chose the TGF- β type II receptor (TGF β RII) as a model target to test adenoviral delivery of both antisense RNAs and shRNAs directed against this receptor. We evaluated whether the efficacy against endogenous TGF β RII could be reliably predicted from a GFP fluorescence screen based on their relative silencing effects on a transiently expressed dicistronic TGF β RII/GFP reporter. Our results show for both antisense RNA and shRNA molecules that their effectiveness in the GFP reporter screen correlated directly with their ability to downregulate exogenous TGF β RII transgene expression. Antisense RNA, however, was ineffective in silencing endogenous TGF β RII. In contrast, all four shRNA constructs were able to downregulate endoge-

nous TGF β RII. The shRNA that demonstrated the most pronounced effect on the TGF β RII/GFP reporter consistently had the most pronounced effect on endogenous TGF β RII in the cell lines tested. These findings have significant implications for the optimization and utility of antisense RNA and shRNA in gene discovery and therapy, as will be discussed.

MATERIALS AND METHODS

Cell culture and transient transfection

Human cell lines 293, A549, and HeLa (ATCC, Rockville, MD) were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT). Transient transfection of A549 cells and 293 cells was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's specifications. The cells were seeded in 24-well plates at 1.5×10^5 cells/well and transfected 24 hours later using 2 μ L Lipofectamine 2000 and 1.2 μ g plasmid DNA. After overnight incubation, the transfection mixture was replaced by growth medium, and the cells were allowed to recover for 24 hours prior to analysis of gene expression. pcDNA-secreted alkaline phosphatase (SEAP) was included in some transfections, so that SEAP activity could be used to normalize for transfection efficiency. SEAP activity was measured as described in Durocher et al. (2000).

Generation of senseTGF β RII/GFP reporter adenovirus (AdCMV5-senseTGF β RII-DC-GFP)

The human TGF β RII cDNA sequence, including 5'-UTR and full-length 3'-UTR, was cloned in the sense direction into the adeno transfer vector pAdCMV5-DC-GFP harboring a dicistronic cassette (Massie et al., 1998; Mosser et al., 1997). The cDNA sequence consists of nucleotide positions from 1237 to 5756 (TGF β RII cDNA HTR2-3, accession No. D50683), except the 5'-UTR from position 1237 to the AUG codon (position 1573) corresponds to the 5'-UTR sequence of the TGF β RII cDNA H2-3FF (positions 1–335, accession No. M85079). This 5'-UTR was detected ubiquitously in both normal and cancerous cell lines (Ogasa et al., 1996). The designated positions of 1237–5756, from the HTR2-3 cDNA sequence, were retained for clarity (Fig. 1). The resulting cassette places the TGF β RII sequence downstream from the AdCMV5 promoter, followed by an internal ribosomal entry site (IRES) and the GFP cistron. The resulting construct was linearized, purified, and cotransfected with Ad5/ Δ E1 Δ E3 viral DNA into 293 cells to generate recombinant adenovirus AdCMV5-senseTGF β RII-DC-GFP, according to the protocol described by Massie et al. (1998).

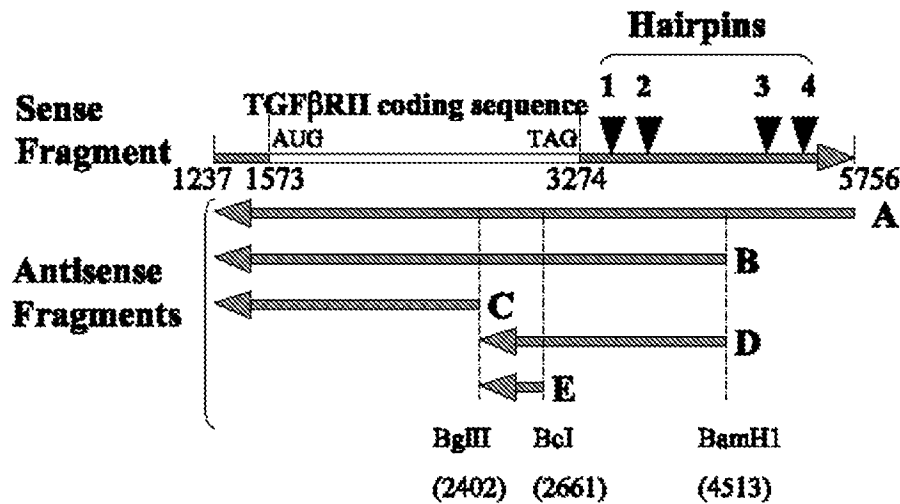


FIG. 1. TGF β RII restriction fragments used to construct the sense discistronic reporter and the antisense adenoviral clones, and the positions of the small hairpins used for shRNA. The top fragment shows the size and orientation of the sense fragment (nucleotides from positions 1237 to 5756 of the reported human TGF β RII cDNA sequence, accession No. D50683). The location (open bar) and position of the TGF β RII coding sequence also are indicated. Antisense fragment A is the same sequence as above but in the reverse orientation. The restriction sites used to generate subfragments B, C, D, and E are shown at the bottom, along with their respective positions (in parentheses). The approximate locations of the small hairpins, numbered 1, 2, 3, and 4, are indicated by the arrowheads. The exact positions and sequences of the hairpins are detailed in Materials and Methods.

Generation of adenoviral antisense TGF β RII fragment clones

Full-length TGF β RII cDNA, as described (labeled Fragment A in Fig. 1), and four subfragments (B, C, D, and E) generated by restriction enzyme digestion (Fig. 1) were cloned in the antisense orientation downstream of the CMV5 promoter in pAdCMV5-DC-GFP. Plasmid DNA was linearized, purified, and cotransfected with Ad5/ Δ E1 Δ E3 viral DNA in 293 cells to generate adenoviral recombinants. GFP-positive viral plaques were collected and amplified separately as small viral stocks in 293 cells.

The identity of the cloned TGF β RII antisense fragment in the selected viral clones was determined by PCR amplification using forward primer F1: 5'-TCTCTC-CACAGGTGTCCAC-3' complementary to the CMV5 promoter site, and reverse primer R1: 5'-ACACCGGC-CTTATTCCAAG-3' complementary to the IRES situated downstream from the antisense insert. PCR amplification was carried out with the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol.

GFP reporter screening assay for adenoviral antisense clones

HeLa cells were plated in 96-well plates at 1×10^5 cells/well in 100 μ L complete DMEM and coinfectd with AdCMV5-senseTGF β RII-DC-GFP (multiplicity of infections [moi] of 100 plaque-forming units [PFU]/cell)

and 10 μ L of the amplified viral antisense clones (moi of approximately 100 PFU/cell) in a final total volume of 200 μ L DMEM. GFP fluorescence was measured 96 hours postinfection using a FluorImager and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Recombinant adenovirus AdCMV5-BFP expressing blue fluorescent protein (BFP) was used as a control in some of the screening experiments (Massie et al., 1998).

Cloning of shRNA

The shRNA sequences were selected according to Tuschl et al. (2002) and were at the following nucleotide positions in the 3'-UTR of the TGF β RII cDNA: position 3308 for hp1, position 3347 for hp2, position 4807 for hp3, and position 5432 for hp4. shRNAs, consisting of 19-bp strands separated by a 9-bp loop, were generated by a single round of PCR amplification according to the protocol of Castanotto et al. (2002). Plasmid pTZU6 (Castanotto et al., 2002), containing the human U6 promoter, was used as a template. The forward primer (U6F) used in all reactions annealed to the 5'-end of the U6 promoter (shown in bold italics): 5'-CGCCGGATCCAAG-**GTCTGGGCAGGAAGAG**-3'.

All reverse primers had an invariable 3'-end complementary to the 3'-end of the U6 promoter (shown in bold), followed by the hairpin sequence (underlined) including a 9-bp loop, six A bases, and a 12-bp spacer sequence containing an SalI restriction site:

sh1R: 5'-ACCTTCGTCGACAAAAAAGAGGCTGCC-
CCTCTCACCATCTCTTGAATGGTGAGAGGGG-
CAGCCTCGGTGTTTCGTCCTT-3'; sh2R: 5'-ACC-
TTCGTCGACAAAAAAGCTGCCCTGAACTGA
TGCTCTCTTGAAGCATCAGTTCAGGGG-
CAGCGGTGTTTCGTCCTT-3'; sh3R: 5'-ACCTTC-
GTCGACAAAAAAGGGTCTCAGTTAGCCCAAG
TCTCTTGAACCTGGGCTAACTGAGACCCGGT-
GTTTCGTCCTT-3'; sh4R: 5'-ACCTTCGTCGAC-
AAAAAAGATTCAAGAGTATTCTCACTCTC-
TTGAAGTGAGAATACTCTTGAATCGGTGTTTC
GTCCTT-3'; GFPshR: 5'-ACCTTCGTCGACAAA-
AAAGACACGTGCTGAAGTCAAGTCTCTTGA-
CTTGACTTCAGCACGTGTCGGTGTTTCGTC-
CTT-3'.

All reactions were carried out within 20 cycles using the Expand High Fidelity PCR System according to the manufacturer's protocol. The PCR products were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI). TOP10 One Shot chemically competent cells (Invitrogen Life Technologies) were used to generate the shRNA plasmids. Positive clones were identified by BamHI/SalI digestion and confirmed by automated sequencing analysis.

Construction of TGF β RII hairpin adenoviral recombinants

The U6 promoter-hairpin sequences hp1, hp2, hp3, hp4, and hpGFP were excised from the plasmid vector with EcoRI, blunt ended using T4 DNA polymerase, and recloned into the XbaI site of adeno transfer vector pAdPS-CMV5-red fluorescent protein (RFP) (Gagnon et al., unpublished observations). This vector also expresses RFP, driven by the CMV5 promoter, and adenoviral protease, driven by the adenoviral major late promoter, to facilitate rapid selection of viral recombinants (Elahi et al., 2002). The vector was linearized and recombined with Ad5 Δ protease (as detailed in Ogorelkova et al., 2003) to generate viral recombinants expressing the individual hairpin sequences.

Flow cytometry analysis of GFP and RFP expression

GFP and RFP emissions were analyzed with an EPICS XL-MCL flow cytometer (Coulter, Miami, FL) equipped with a 15-mW argon ion laser and the following filters: 488-nm laser blocking, 488-nm long-pass dichroic, 550-nm long-pass dichroic, and 525-nm band-pass. The data collection was done using Multiparameter Data Acquisition and Display System (MDADS, Coulter). GFP expression was quantified by fluorescence index (percent of GFP-positive cells \times mean fluorescence).

RNA extraction and quantitative RT-PCR analysis

Total cellular RNA was prepared using TRIzol reagent (Invitrogen Life Technologies). Prior to first-strand cDNA synthesis, DNA contamination was eliminated by DNase I treatment. RNA (2 μ g) was incubated with 1 U DNase I, amplification grade according to manufacturer's instructions and then used for first-strand cDNA synthesis with 4 pmol of each β -actin-specific primer (5'-GTACTTGCCTCAGGAGGAG-3') and TGF β RII-specific primer (5'-CAGAAGCTGGGAATTCTGG-3'). Reverse transcription was carried out with reverse transcriptase AMV (Roche Molecular Biochemicals) according to the manufacturer's protocol. Quantitative real-time PCR was performed on a LightCycler instrument (Roche) using LightCycler FastStart DNA Master SYBR Green I (Roche). Quantities μ L and 5 μ L of 1:10 diluted RT reaction were used for actin and TGF β RII cDNA amplification, respectively. The reactions were carried out in 10 μ L with 5 pmol of each forward and reverse primer, 3 mM MgCl₂, and 1 μ L FastStart Reaction/FastStart Enzyme Mix. The PCR primer sequences were as follows: actin-F: 5'-TCCCTGGAAGAGCTACGA-3'; actin-R: 5'-AGCACTGTGTTGGCGTACAG-3'; TGF β RII-F: 5'-TAACCTGCTGCTGTGTGAC-3'; TGF β RII-R: 5'-CAACACGTTGTCTTCATGC-3'. Negative controls for RT and PCR were included in each experiment. Crossing points (CP) for each transcript were determined using the Fit Point Method (LightCycler software 3.3, Roche). The relative quantification of TGF β RII gene expression for each sample vs. control in comparison to β -actin as a reference gene was performed using the equation:

$$R = 2^{\Delta CP_{TGF\beta RII}(\text{control-sample}) / 2^{\Delta CP_{actin}(\text{control-sample})}}$$

where R is the relative expression ratio of TGF β RII gene, Δ CP is the crossing points deviation of control minus sample, and 2 corresponds to an approximate PCR efficiency (Pfaffl, 2001; Rasmussen, 2001).

Western blot detection

Reducing SDS-PAGE was performed on commercial 12% tris-glycine Gels (Novex, Invitrogen Life Technologies). SDS-PAGE and immunoblotting were carried out according to the manufacturer's protocol. Polyclonal antibodies sc220, sc398, and sc03, specific for TGF β RII, TGF β RI, and epidermal growth factor receptor (EGFR), respectively, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody against phospho-Ser51-eukaryotic initiation factor 2 α (eIF2 α) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antiactin antibody was purchased from ICN Biomedicals (Aurora, OH).

Cross-linking of ^{125}I -TGF- β 1 to TGF- β receptors

A549 cells were seeded onto 6-well plates (1×10^5 cells/well). After infection with either antisense or shRNA viruses, the cells were treated with 200 pM ^{125}I -TGF- β 1 (Perkin-Elmer Life Sciences, Woodbridge, ON) at 4°C for 2.5 hours, followed by the addition of cross-linking agent BS³ (Pierce Chemical Co., Rockford, IL) to specifically label the TGF- β receptors on the cell surface (as described by Mitchell and O'Connor-McCourt, 1991). Cell lysates were prepared, and the receptors were separated by SDS-PAGE and then detected by a phosphorimager and quantified using ImageQuant software (Molecular Dynamics).

HeLa CAGA-luciferase reporter cell assay

HeLa cells were stably transfected with a luciferase reporter driven by Smad3/4 binding elements (CAGA) (Dennler et al., 1998). For reporter gene assays, the cells were plated at 5×10^4 cells/well in a 24-well plate and infected the next day with hairpin adenovirus at an moi of 100–200. Four days after infection, the cells were left untreated or treated with 100 pM TGF- β 1 (R&D Systems, Minneapolis, MN) overnight at 37°C. The cells were harvested into 100 μL reporter lysis buffer, and the luciferase activity was measured as recommended by the manufacturer (Dual-Luciferase Reporter Assay System, Promega) using an MLX microtiter plate luminometer (Dy nex Technologies Inc., Chantilly, VA). All luciferase assays were performed in triplicate and normalized to protein content in the lysates.

RESULTS

Screening of adenoviral antisense TGF β R2 clones using TGF β R2/GFP reporter

Five defined TGF β R2 restriction fragments, A, B, C, D, and E, were cloned separately in the viral vector pAd-CMV5-DC-GFP (Massie et al., 1998) in the antisense orientation (Fig. 1). The antisense effect of the antisense adenoviral clones was tested on a virally delivered, dicistronic sense TGF β R2/GFP reporter in a screening assay performed in microtiter plates (Fig. 2A). In the assay, HeLa cells were coinfect ed with the sense TGF β R2/GFP-expressing and antisense TGF β R2/GFP-expressing viruses, followed by measurement of GFP fluorescence 96 hours postinfection. Coinfection of sense TGF- β R2/GFP virus with the antisense clones reduced the GFP fluorescence from approximately 2-fold (clones A, B, and D) to 13-fold (clone C) compared with control cells coinfect ed with adenovirus expressing BFP. In contrast, clone E scored negative in the assay. The observed increase in the GFP fluorescence for clone E above the level for the BFP control was likely due to the cumulative expression of GFP from both sense and antisense adenoviruses, as clone E, when expressed alone, displayed a higher GFP fluorescence than the sense construct (data not shown). We confirmed the antisense efficacies by Western blotting of lysates prepared from A549 cells coinfect ed with sense TGF β R2/GFP virus and the antisense clones, using an antibody probe specific for TGF β R2 (Fig. 2B). The relative decrease in TGF β R2 transgene expression caused by each viral clone corre-

FIG. 2. Analysis of the effect of antisense virus clones on the TGF β R2/GFP reporter and TGF β R2 expression. (A) GFP fluorescence screening of the adenoviral antisense clones. The bars show GFP fluorescence levels, measured 96 hours postinfection, for HeLa cells coinfect ed with sense TGF β R2/GFP virus and the indicated antisense TGF β R2/GFP virus clone. The average values (\pm SE) for three independent experiments are shown. Cells coinfect ed by sense TGF β R2/GFP virus and adenovirus expressing BFP serve as a negative control. (B) Western blot detection of the TGF β R2 transgene protein. Western blotting was performed on lysates collected 48 hours postinfection of A549 cells coinfect ed with sense TGF β R2/GFP virus (moi 100) and the indicated antisense TGF β R2/GFP viral clones (moi 100). Mock indicates cells that were not infected. The blots were probed with antibodies specific for either TGF β R2 or phospho-Ser51-eIF2 α (P*-eIF2 α), as indicated at right. An antibody specific for β -actin was also used to control for protein loading. (C) Specificity of the adenovirus antisense TGF β R2 clones for TGF β R2 vs. TGF β R1. 293 cells were transfected with a plasmid expressing either TGF β R2 (top) or TGF β R1 (bottom), followed by infection with the indicated antisense TGF β R2 viral clone or antisense GFP virus. Protein lysates were prepared, and equal protein amounts were subjected to SDS-PAGE, blotted, and probed with antibodies specific for TGF β R2 or TGF β R1. (D) Specificity of the adenovirus antisense TGF β R2 clone A for TGF β R2 vs. EGFR. A549 cells were infected with TGF β R2 sense virus, virus expressing EGFR, and TGF β R2 antisense A virus, as indicated. Protein lysates were prepared and equal protein amounts were subjected to SDS-PAGE, blotted, and probed with antibodies specific for TGF β R2 (bottom) or EGFR (top). (E) Endogenous TGF β R2 protein levels in A549 cells infected by selected antisense viral clones. The cells were infected for 48 hours (moi 200), followed by receptor binding and cross-linking of ^{125}I -TGF- β 1. Cell lysates were prepared and subjected to SDS-PAGE. Radioactively labeled TGF- β R2 was detected and quantified using a phosphorimager. TGF β R2 band intensities were standardized relative to TGF β R3, whose levels are not affected by antisense TGF β R2 virus. (F) Endogenous TGF β R2 transcript levels in A549 cells infected with antisense viral clones C and E. The level of the TGF β R2 transcript was determined by real-time RT-PCR relative to control cells infected by virus expressing BFP. The cells were infected for 36 hours (moi 200), followed by extraction of total RNA. The quantification of TGF β R2 transcripts was performed for each sample in comparison to β -actin as a reference gene. Average values \pm SE from three independent experiments are shown.

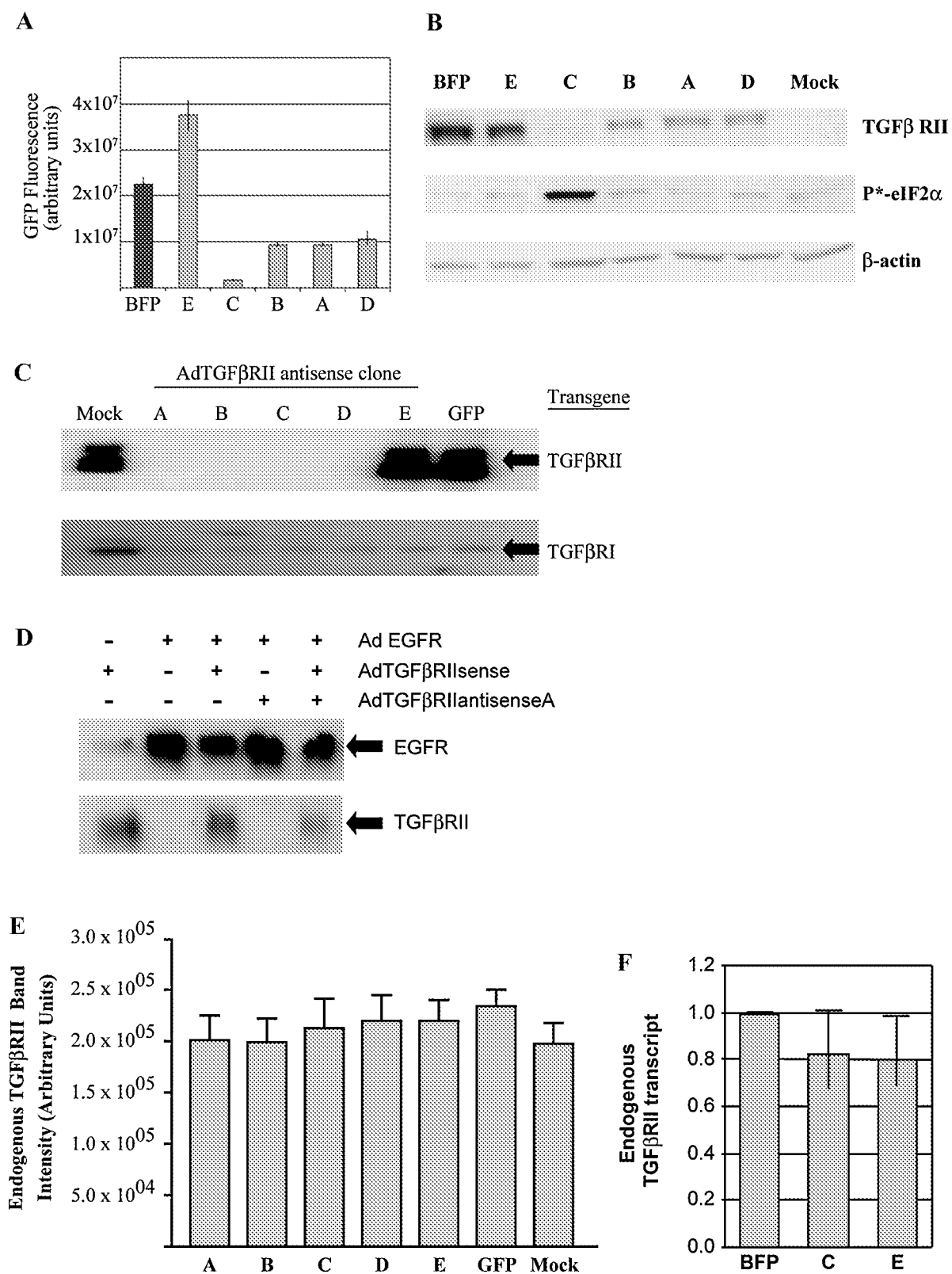


FIG. 2.

lated directly with its effect on the GFP reporter (compare Fig. 2A and 2B), with antisense clone C causing the most dramatic decrease and clone E having little or no effect. These results demonstrate the validity of using the TGF β RII/GFP reporter to screen for optimal antisense fragments.

Duplex RNA molecules in the cytoplasm of mammalian cells can trigger nonspecific suppression of gene expression mediated in part through a dsRNA-activated protein kinase (PKR) pathway (reviewed in Kumar and Carmichael, 1998). Activated PKR, in turn, phosphorylates a number of substrates, including eIF2 α , leading to inhibition of protein synthesis. Therefore, in parallel to our analyses of transgene expression, we performed immunodetection of phosphorylated eIF2 α to determine whether PKR activation contributed to the observed antisense effect (Fig. 2B). We found an increased level of eIF2 α phosphorylation for cells coinfecting with the most efficient antisense virus (clone C), whereas the less efficient antisense viruses (clones A, B, and D) did not detectably promote eIF2 α phosphorylation. Despite PKR activation, the specificity of clone C for TGF β RII, as well as clones A, B, and D, is evidenced by their ability to reduce TGF β RII transgene expression in 293 cells, whereas none of these viral clones reduced another receptor, TGF- β receptor type I (TGF β RI) (Fig. 2C). In A549 cells infected with both TGF β RII sense virus (A fragment) and a virus encoding EGFR, TGF β RIIantisense virus clone A reduced TGF β RII protein expression but had no effect on the EGFR (Fig. 2D, compare lane 5 with control lane 3). Similar results were obtained for TGF β RIIantisense virus clones B, C, and D (data not shown). Taken together, these results indicate that although dsRNA-induced effects are evoked in some cases, the antisense viruses promote a TGF β RII-specific silencing effect that is not simply due to a global shutdown of protein synthesis. The apparently similar effectiveness of antisense clones A, B, C, and D for silencing TGF β RII in 293 cells (Fig. 2C) may be a result of higher expression levels of the antisense transcripts in this cell line, following adenoviral replication, compared with A549 cells (Fig. 2B).

Evaluation of antisense RNA efficacy on endogenous TGF β RII

The silencing efficiency of virally expressed antisense fragments on endogenous TGF β RII protein in A549 cells was analyzed by cross-linking experiments using ¹²⁵I-TGF- β 1 to radioactively label and quantify the level of this receptor on the cell surface (Fig. 2E). This method of detecting TGF β RII was used because the level of endogenous TGF β RII is below the sensitivity of Western blots. TGF β RII expression was normalized relative to another TGF- β receptor, TGF β RIII, which can bind

TGF- β independently of TGF β RII. As shown in Figure 2E, none of the antisense viruses induced a detectable decrease in endogenous TGF β RII. Viral clones C and E, having the maximum and minimum effects, respectively, on the TGF β RII transgene, were further analyzed for their effects on endogenous TGF β RII at the transcript level using quantitative RT-PCR (Fig. 2F). The quantification was specific for the endogenous sense transcript, as the TGF β RII sequence was amplified using sense-specific primers that cannot hybridize with the antisense viral transcripts. Clone C and clone E did not produce a statistically significant decrease in the endogenous target. Thus, our results show that the different silencing efficiencies observed for these viral clones on the exogenous TGF β RII transgene could not be duplicated with endogenous TGF β RII at either the transcript or protein level.

Cloning of shRNAs and GFP reporter-based screen for RNAi efficiency

As an alternative to antisense RNA-mediated silencing, we generated plasmid constructs expressing four distinct shRNAs targeting human TGF β RII and driven by the human U6 promoter. The target sites were selected to be in the 3'-UTR of the TGF β RII mRNA, as this region is not highly conserved among other TGF- β receptors. The positions of these target sites (numbered 1–4) are shown in Figure 1. A control shRNA was also designed to specifically target GFP (hpGFP).

RNAi efficiency was first tested by cotransfection of the shRNA plasmids individually with the sense TGF β RII/GFP reporter plasmid in readily transfectable 293 cells. All four shRNAs had at least a 3-fold inhibitory effect on GFP expression relative to cells transfected with empty vector pTZU6 (Fig. 3A). The reduction in GFP fluorescence correlated directly with the effects of these shRNAs on expression of the TGF β RII transgene, as determined by Western blotting (Fig. 3B). Notably, hp4 had the most pronounced effect in both these assays, resulting in approximately 5% residual GFP fluorescence and almost complete elimination of protein expression. In these assays, the hairpin directed against GFP (hpGFP) also caused a reduction in TGF β RII expression, as expected, because of its RNAi targeting effect on the dicistronic transcript, which has both TGF β RII and GFP open reading frames (ORFs).

The capacity of the shRNA plasmids to downregulate expression of the endogenous TGF β RII transcript was tested by transient transfection of the TGF β RII shRNA constructs in 293 cells, followed by quantitative RT-PCR (Fig. 3C). All four shRNAs reduced TGF β RII expression, whereas hpGFP, in this case, had no effect, confirming the specificity of the TGF β RII shRNAs for targeting the endogenous transcript. Consistent with its maximal effect against the exogenous TGF β RII trans-

gene (Fig. 3A,B), hp4 was also most efficient in silencing endogenous TGF β RII (Fig. 3C).

Analysis of efficiency of adenovirus-delivered hairpin RNAs on silencing endogenous TGF β RII

In order to more effectively express these hairpins in epithelial cells, such as A549 cells, which tend to be poorly transfected, the U6 promoter-shRNA cassettes, encoding three selected TGF β RII hairpins, hp1, hp3, and hp4, and the control hpGFP, were recloned separately into adeno transfer vector pAdPS-CMV5-RFP. In addition to the protease expression cassette used for selection of adenoviral recombinants, this vector also contains a cassette expressing RFP that is driven by a CMV5 promoter, to facilitate monitoring of gene transfer (D. Gagnon et al., unpublished observations; Ogorelkova et al., 2003). The levels of RFP correlated directly with the respective moi (as estimated by plaque formation on 293 cells) for all the hairpin recombinants (data not shown). The silencing efficiency of the TGF β RII hp viruses was first tested on the exogenous TGF β RII delivered by adenoviral infection in A549 cells. The results shown in Figure 3D confirmed that all TGF β RII hp viruses downregulated the expression of exogenous TGF β RII (lanes 6, 8, and 10), albeit less efficiently than the antisense TGF β RII clone C (lane 3). The activation of the PKR-mediated pathway was also examined by assessing the phosphorylation level of eIF2 α . Whereas hp3 and hp4 did not evoke any increase in eIF2 α phosphorylation, hp1 reproducibly increased eIF2 α phosphorylation (Fig. 3D, middle, lane 6). However, this increase was relatively modest compared with antisense TGF β RII clone C.

The silencing efficiency of the TGF β RII hp viruses was then tested on endogenous TGF β RII in A549 cells. The level of endogenous TGF β RII was assessed directly, following viral infection, by receptor cross-linking experiments using ¹²⁵I-TGF- β 1 (Fig. 3E). All three TGF β RII hp viruses significantly reduced expression of TGF β RII, compared with cells infected by RFP or hpGFP viruses. These hairpin effects were specific for TGF β RII, as no reduction was seen for TGF β RIII. The intensity of the RII band in each lane was quantified, and the average level was determined for two separate experiments and shown as a percentage relative to control RFP-virus-infected cells (Fig. 3F). This analysis indicates that all three hairpins reduce RII levels by >50%, compared with the RFP-virus control. The slight reduction in RII observed for hpGFP, relative to RFP-virus-infected cells, indicates that nonspecific effects of virus-delivered hairpins may occur in A549 cells.

The hairpin viruses were also tested on HeLa cells stably expressing a luciferase reporter gene driven by the TGF- β -responsive CAGA-promoter (Fig. 3G). An effec-

tive hairpin should silence TGF β RII in these cells and, hence, abrogate TGF- β signaling and luciferase expression. The results in Figure 3G show that all three hairpin viruses markedly reduced luciferase levels compared with the mock and RFP virus-infected control cells. It is noteworthy that the virus expressing hpGFP did not reduce luciferase levels, confirming that the TGF β RII hp viruses specifically target TGF β RII. Taken together, our data indicate that in contrast to the virus-delivered antisense RNA, all three TGF β RII hp viruses can target endogenous TGF β RII, albeit with slightly different silencing efficiencies.

DISCUSSION

In the present study, our goal was to assess the efficacy of antisense RNAs and shRNAs to downregulate expression of TGF β RII. To facilitate the selection of highly efficient silencing molecules, we first tested the efficacy of adenoviral antisense RNAs and shRNAs on a transiently expressed dicistronic TGF β RII/GFP reporter using GFP-based assays. Our results showed that four of the five tested antisense fragments downregulated the TGF β RII transgene with the same magnitude as the coexpressed GFP reporter, validating the reporter assay for selection of optimal antisense RNA fragments. We were surprised, however, that none of these antisense RNAs significantly affected endogenous TGF β RII expression. This discrepancy may be explained by differential compartmentalization between the virus-delivered TGF β RII and endogenous TGF β RII sense transcripts. Antisense efficiency is influenced not only by mRNA structural constraints but also by appropriate localization of sense and antisense strands in the same cellular compartment (Sczakiel and Far, 2002; Lehmann et al., 2000; Kumar and Carmichael, 1997). Adenovirus-delivered sense and antisense RNAs are likely to be colocalized during their transcription from the viral genomes in the nucleus, thus promoting hybridization. In addition, based on the available TGF β RII cDNAs, there is evidence for variable splicing in the 5'-UTRs (Ogasa et al., 1996). The sense TGF β RII/GFP reporter transcript used in our assay may lack 5'-UTR sequences (i.e., upstream of nucleotide position 1237) (Fig. 1) that potentially contain localization motifs that direct the native transcript to a cellular location distinct from that of the antisense RNA.

Viral delivery of antisense RNA has, nevertheless, proven to be a viable strategy for multiple endogenous targets, including the gene for TGF- β 1 ligand and hsp70 (Arias et al., 2002; Gabai et al., 2000; Gondi et al., 2003; Haberman et al., 2002; Li et al., 2003; Nakano et al., 2001; Ohnami et al., 2003; Zhang et al., 2003). It is noteworthy that in the case of hsp70, there was a perfect correlation between the magnitude of downregulation resulting from

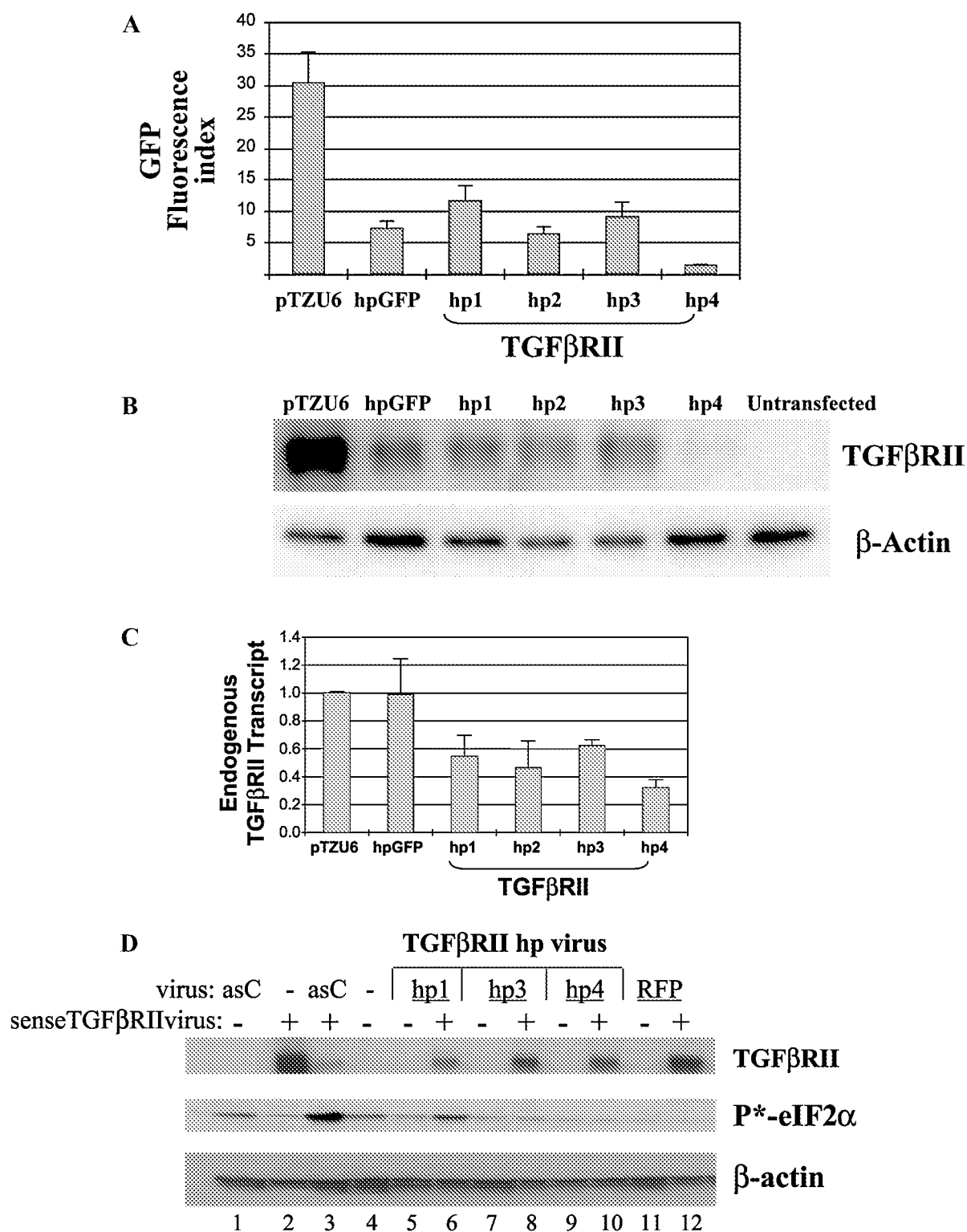
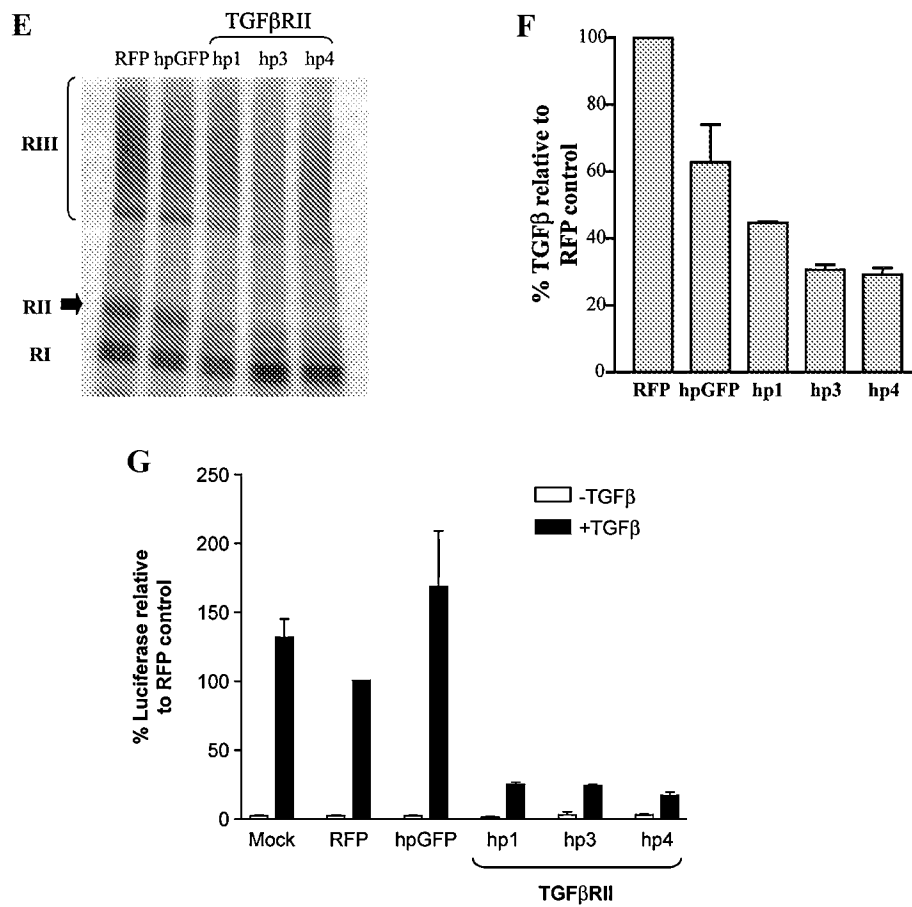


FIG. 3. Effect of TGFβRII hairpins on the TGFβRII/GFP reporter and TGFβRII expression. (A) Flow cytometry analysis of GFP expression in 293 cells cotransfected with plasmid expressing dicistronic sense TGFβRII/GFP and the indicated shRNA plasmid. An additional cotransfected plasmid pSEAP2-control, expressing secreted alkaline phosphatase, was included in each case to normalize the GFP fluorescence index (% GFP-positive cells × mean fluorescence) relative to transfection efficiency. Cells cotransfected with empty vector pTZU6 served as a control. Average values ± SE from three independent experiments are shown. (B) Western blot detection of TGFβRII transgene expression in 293 cells cotransfected with plasmid expressing sense TGFβRII/GFP and the indicated hairpin plasmid. Western blots were probed with antibody specific for TGFβRII or β-actin, as indi-



cated at right. Untransfected cells or cells cotransfected with pTZU6 served as negative and positive controls, respectively. (C) Effect of transfected TGF β RII hairpin constructs on the endogenous TGF β RII transcript in 293 cells. The level of the TGF β RII transcript was determined by real-time RT-PCR relative to cells transfected with pTZU6. In each case, the cells were transfected with the indicated shRNA plasmids and cotransfected with plasmid pDsRed2-N1 expressing RFP to standardize for transfection efficiency (i.e., % RFP-positive cells). Total RNA was extracted 36 hours posttransfection. Average values \pm SE from three independent experiments are shown. (D) Effect of TGF β RII hp viruses on eIF2 α phosphorylation. A549 cells were infected with the indicated TGF β RII hp viruses, with (+) or without (–) coinfecting sense TGF β RII virus. Cells infected with antisense TGF β RII virus clone C (asC) or nonhairpin RFP virus (RFP) were included as controls. Cell extracts were prepared, followed by Western blotting using antibody probes for TGF β RII, phosphorylated eIF2 α (P*-eIF2 α), and β -actin. (E) Effect of adenovirus-delivered TGF β RII hairpins on endogenous TGF β RII protein in A549 cells. A549 cells were infected with parental virus (RFP) or adenoviruses expressing the indicated hairpins (moi 200). Five days postinfection, the surface TGF- β receptors were radioactively labeled by cross-linking to 125 I-TGF- β at 4°C. Cell lysates were prepared and equal protein amounts were electrophoresed on gradient SDS-PAGE. TGF β RIII (which runs as a smear due to extensive glycosylation), TGF β RII, and TGF β RI were detected by a phosphor-imager, and their positions are indicated at left. (F) Relative endogenous TGF β RII levels in A549 cells infected by the hpGFP or TGF β RII hairpin adenoviruses. The band intensity of TGF β RII was quantified for two separate cross-linking experiments, which were performed in duplicate. The graph shows the average TGF β RII level (\pm SD) expressed as a percentage relative to the parental RFP virus control. (G) Effect of adenovirus-delivered TGF β RII hairpins on TGF- β -induced luciferase response in HeLa CAGA cells. HeLa CAGA cells, stably expressing a TGF- β -responsive luciferase reporter, were infected with adenovirus (moi 200) expressing the TGF β RII hairpins (hp1, hp3, or hp4) or a hairpin for GFP (hpGFP). Cells that were mock infected or infected with parental virus (RFP) were included as controls. Five days postinfection, the cells were treated with (open bars) or without (black bars) 100 pM TGF- β 1 for 18 hours, followed by preparation of cell lysates and measurement of luciferase activity. The graph shows the average luciferase levels (\pm SD), expressed as percentages relative to the RFP control, determined for four separate experiments performed in triplicate.

antisense RNA expression on both virus-delivered exogenous hsp70 coexpressed with GFP, and endogenous hsp70 (Gabai et al., 2000; unpublished observations). Furthermore, in a recent study using a transgene/GFP reporter screen, we successfully selected, from an adenovirus library, antisense RNAs with greater silencing activities toward the insulin growth factor (IGF)-1 receptor in a variety of tumor cells (unpublished observations). One caveat that our study revealed is the potential activation of PKR that can lead to nonspecific reduction of protein expression as seen for our most efficient construct, clone C (Fig. 2B). This could ultimately bias assessment of the specific downregulation on the endogenous target. Although not as well documented, this can also happen with shRNAs, as recently reported (Bridge et al., 2003; Sledz et al., 2003; Kim et al., 2004) and as we have shown for one of our hairpin constructs (Fig. 3D). Nonetheless, it is possible that activation of PKR requires a fairly high level of sense and antisense dsRNA as obtained after transfection of the sense construct. Should that be the case, this may not represent a significant problem when only low levels of endogenous mRNA are expressed.

We also took advantage of the TGF β RII/GFP reporter approach as an initial assessment of shRNAs directed against TGF β RII. In this case, we transiently transfected 293 cells with four different shRNA plasmids and assessed their ability to reduce GFP fluorescence using flow cytometry. All four shRNAs scored positive for GFP reduction and were able to reduce expression of both transfected and endogenous TGF β RII in 293 cells (Fig. 3A,B,C). The most efficient hairpin among the four tested with the TGF β RII/GFP reporter, hp4, was also the most consistent in silencing endogenous TGF β RII in all the cell lines tested. Our results, therefore, demonstrate the relevance of using the TGF β RII/GFP reporter to identify the best candidate hairpin for TGF β RII.

In summary, our results show that whereas both shRNA and antisense RNA can efficiently reduce exogenously expressed TGF β RII, only shRNA was able to target endogenous TGF β RII. This stands in contrast to other endogenous gene products, some of which we have mentioned, that can be silenced using antisense technology. The success of shRNA vs. antisense RNA in targeting endogenous TGF β RII might be attributed to differences in their underlying modes of action or access to the endogenous TGF β RII transcript. Further work is needed to address the fundamental differences in the accessibility of antisense RNAs and shRNAs to their endogenous targets, including an assessment of different promoters used to drive these silencing molecules.

ACKNOWLEDGMENTS

This work was supported by the Genome and Health Initiative Program of the National Research Council

(NRC) to B.M. and M.O.M., a Génome Québec Consortium grant to B.M., a Stemcell Network Center of Excellence grant to B.M., and a National Cancer Institute of Canada operating grant to Pnina Brodt, Jacques Gali-peau, and B.M. This is an NRC publication No. 47756.

REFERENCES

- ABBAS-TERKI, T., BLANCO-BOSE, W., DEGLON, N., PRALONG, W., and AEBISCHER, P. (2002). Lenti-viral-mediated RNA interference. *Hum. Gene Ther.* **13**, 2197–2201.
- ARIAS, M., LAHME, B., VAN DE LEUR, E., GRESSNER, A.M., and WEISKERCHEN, R. (2002). Adenoviral delivery of an antisense RNA complementary to the 3' coding sequence of transforming growth factor- β 1 inhibits fibrogenic activities of hepatic stellate cells. *Cell Differ.* **13**, 265–273.
- BLOBE, G.C., SCHIEMANN, W.P., and LODISH, H.F. (2000). Role of transforming growth factor β in human disease. *N. Engl. J. Med.* **342**, 1350–1358.
- BRIDGE, A.J., PEBERNARD, S., DUCRAUX, A., NICOULAZ, A.L., and IGGO, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**, 263–264.
- BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553.
- CASTANOTTO, D., LI, H., and ROSSI, J.J. (2002). Functional siRNA expression from transfected PCR products. *RNA* **8**, 1454–1460.
- DENNLER, S., ITOH, S., VIVIEN, D., TEN DIJKE, P., HUET, S., and GAUTHIER, J.M. (1998). Direct binding of Smad3 and Smad4 to critical TGF β -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* **17**, 3091–3100.
- DING, Y., and LAWRENCE, C.E. (2001). Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond. *Nucleic Acids Res.* **29**, 1034–1046.
- DUROCHER, Y., PERRET, S., TIBAudeau, E., GAUMOND, M.H., KAMEN, A., STOCCO, R., and ABRAMOVITZ, M. (2000). A reporter gene assay for high-throughput screening of G-protein coupled receptors stably or transiently expressed in HEK293 EBNA cells grown in suspension culture. *Anal. Biochem.* **284**, 316–326.
- ELAHI, S.M., OUALIKENE, W., NAGHDI, L., O'CONNOR-McCOURT, M., and MASSIE, B. (2002) Adenovirus-based libraries: Efficient generation of recombinant adenoviruses by positive selection with the adenovirus protease. *Gene Ther.* **18**, 1238–1246.
- ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.
- GABAI, V.L., VOLLOCH, V., YAGLOM, J.A., MERIIN, A.B., KOUTROUMANIS, M., MASSIE, B., MOSSER, D.D., and SHERMAN, M.Y. (2000). Hsp72-mediated suppression of stress-kinase JNK is implicated in development

- of tolerance to caspase-independent cell death. *Mol. Cell. Biol.* **20**, 6826–6836.
- GONDI, C.S., LAKKA, S.S., NIRANJAN, Y., SIDDIQUE, K., DINH, D.D., OLIVERO, W.C., GUJRATI, M., and RAO, J.S. (2003). Expression of antisense uPAR and antisense uPA from a bicistronic adenoviral construct inhibits glioma cell invasion, tumor growth, and angiogenesis. *Oncogene* **22**, 5967–5975.
- HABERMAN, R.P., CRISWELL, H.E., SNOWDY, S., MING, Z., BREESE, G.R., SAMULSKI, R.J., and McCOWN, T.J. (2002). Therapeutic liabilities of *in vivo* viral vector tropism: Adeno-associated virus vectors, NMDAR1 antisense and focal seizure sensitivity. *Mol. Ther.* **6**, 495–500.
- HOLEN, T., AMARZGUIOU, M., WIIGER, M.T., BABAIE, E., and PRYDZ, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.* **30**, 1757–1766.
- KIM, D.H., LONGO, M., HAN, Y., LUNDBERG, P., CANTIN, E., and ROSSI, J.J. (2004). Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat. Biotechnol.* **22**, 321–325.
- KUMAR, M., and CARMICHAEL, G.G. (1997). Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. *Proc. Natl. Acad. Sci. USA* **94**, 3542–3547.
- KUMAR, M., and CARMICHAEL, G.G. (1998). Antisense RNA: Function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol. Mol. Biol. Rev.* **62**, 1415–1434.
- KUMAR, R., CONKLIN, D.S., and MITTAL, V. (2003). High-throughput selection of effective RNAi probes for gene silencing. *Genome Res.* **13**, 2333–2340.
- LAWRENCE, D.A. (1996). Transforming growth factor-beta: A general review. *Eur. Cytokine Netw.* **7**, 363–374.
- LEE, N.S., DOHJIMA, T., BAUER, G., LI, H., LI, M.J., EHSANI, A., SALVATERRA, P., and ROSSI, J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.* **20**, 500–505.
- LEHMANN, M.J., PATZEL, V., and SCZAKIEL, G. (2000). Theoretical design of antisense genes with statistically increased efficacy. *Nucleic Acids Res.* **28**, 2597–2604.
- LI, G.C., HE, F., SHAO, X., URANO, M., SHEN, L., KIM, D., BORRELLI, M., LEIBEL, S.A., GUTLIN, P.H., and LING, C.C. (2003). Adenovirus-mediated heat-activated antisense Ku70 expression radiosensitizes tumor cells *in vitro* and *in vivo*. *Cancer Res.* **63**, 3268–3274.
- MASSAGUÉ, J., BLAIN, S.W., and LO, R.S. (2000). TGF β signaling in growth control, cancer and heritable disorders. *Cell* **103**, 295–309.
- MASSIE, B., MOSSER, D.D., KOUTROUMANIS, M., VITTÉ-MONY, I., LAMOUREUX, L., COUTURE, F., PAQUET, L., GUILBAULT, C., DIONNE, J., CHALA, D., JOLICOEUR, P., and LANGELIER, Y. (1998). New adenovirus vectors for protein production and gene transfer. *Cytotechnology* **28**, 53–64.
- MITCHELL, E.J., and O'CONNOR-McCOURT, M.D. (1991). A transforming growth factor beta (TGF-beta) receptor from human placenta exhibits a greater affinity for TGF-beta 2 than for TGF-beta 1. *Biochemistry* **30**, 4350–4356.
- MIYAGISHI, M., HAYASHI, M., and TAIRA, K. (2003). Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* **13**, 1–7.
- MOSSER, D.D., CARON, A.W., BOURGET, L., JOLICOEUR, P., and MASSIE, B. (1997). Use of a dicistronic expression cassette encoding the green fluorescent protein for the screening and selection of cells expressing inducible gene products. *Biotechniques* **22**, 150–154.
- NAGY, P., ARNDT-JOVIN, D.J., and JOVIN, T.M. (2003). Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells. *Exp. Cell Res.* **285**, 39–49.
- NAKANO, M., AOKI, K., MATSUMOTO, N., OHNAMI, S., HATANAKA, K., HIBI, T., TERADA, M., and YUSHIDA, T. (2001). Suppression of colorectal cancer growth using an adenovirus vector expressing an antisense K-ras RNA. *Mol. Ther.* **3**, 491–499.
- OGASA, H., NOMA, T., MURATA, H., KAWAI, S., and NAKAZAWA, A. (1996). Cloning of a cDNA encoding the human transforming growth factor-beta type II receptor: Heterogeneity of the mRNA. *Gene* **181**, 185–190.
- OGORELKOVA, M., ELHAI, S.M., GAGNON, D., and MASSIE, B. (2003). DNA delivery to cell in culture: Generation of adenoviral libraries for high throughput functional screening. In: *Methods in Molecular Biology entitled Gene Transfer to Mammalian Cells*. W.C. Heiser, ed. (Humana Press, Inc., Totowa, NJ), pp. 15–27.
- OHKAWA, J., and TAIRA, K. (2000). Control of the functional activity of an antisense RNA by a tetracycline-responsive derivative of the human U6 snRNA promoter. *Hum. Gene Ther.* **11**, 577–585.
- OHNAMI, S., AOKI, K., YOSHIDA, K., OHNAMI, S., HATANAKA, K., SUZUKI, K., SASAKI, H., and YOSHIDA, T. (2003). Expression profiles of pancreatic cancer cell lines infected with antisense K-ras-expressing adenoviral vector. *Biochem. Biophys. Res. Commun.* **309**, 798–803.
- PADDISON, P.J., and HANNON, G.J. (2002). RNA interference: The new somatic cell genetics? *Cancer Cell* **2**, 17–23.
- PATZEL, V., and SCZAKIEL, G. (1998). Theoretical design of antisense RNA structures substantially improves annealing kinetics and efficacy in human cells. *Nat. Biotechnol.* **16**, 64–68.
- PFAFFL, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
- RASMUSSEN, R. (2001). Quantification on the LightCycler. In: S. Meuer, C. Wittwer, K. Nakagawara, eds. *Rapid Cycle Real-Time PCR, Methods and Applications*. (Springer Press, Heidelberg), pp. 21–34.
- SCZAKIEL, G. (1997). The design of antisense RNA. *Antisense Nucleic Acid Drug Dev.* **7**, 439–444.
- SCZAKIEL, G., and FAR, R.K. (2002). The role of target accessibility for antisense inhibition. *Curr. Opin. Mol. Ther.* **4**, 149–153.
- SHEN, C., BUCK, A.K., LIU, X., WINKLER, M., and RESKE, S.N. (2003). Gene silencing by adenovirus-delivered siRNA. *FEBS Lett.* **539**, 111–114.
- SHI, Y. (2003). Mammalian RNAi for the masses. *Trends Genet.* **19**, 9–12.
- SLEDZ, C.A., HOLKO, M., DE VEER, M.J., SILVERMAN, R.H., and WILLIAMS, B.R.G. (2003). Activation of the in-

- terferon system by short interfering RNAs. *Nat. Cell Biol.* **5**, 771–772.
- T'HOEN, P.A., ROSEMA, B.S., COMMANDEUR, J.N., VERMEULEN, N.P., MANOHARAN, M., VAN BERKEL, T.J., BIESSEN, E.A., and BIJSTERBOSCH, M.K. (2002). Selection of effective antisense oligodeoxynucleotides with a green fluorescent protein-based assay. Discovery of selective and potent inhibitors of glutathione S-transferase Mu expression. *Eur. J. Biochem.* **269**, 2574–2583.
- TUSCHL, T., ELBASHIR, S., HARBORTH, J., and WEBER, K. (2002). The siRNA user guide, at www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html.
- WEISS, B., DAVIDKOVA, G., and ZHOU, L.W. (1999). Antisense RNA gene therapy for studying and modulating biological processes. *Cell. Mol. Life Sci.* **55**, 334–358.
- YU, J.Y., DERUITER, S.L., and TURNER D.L. (2002). RNA interference by expression of short interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 6047–6052.
- ZHANG, X., CHEN, Z., CHEN, Y., and TONG, T. (2003). Delivering antisense telomerase RNA by a hybrid adenovirus/adeno-associated virus significantly suppresses the malignant phenotype and enhances cell apoptosis of human breast cancer cells. *Oncogene* **22**, 2405–2416.

Address reprint requests to:

Dr. Bernard Massie
Biotechnology Research Institute
6100 Royalmount Avenue
Montréal, Québec
Canada H4P 2R2

E-mail: bernard.massie@cnrc-nrc.gc.ca

Received April 16, 2004; accepted in revised form
January 6, 2006.